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Studies on the Carcinogenicity of the Glucuronides of *N*-Hydroxy-2-acetylaminofluorene and *N*-2-Fluorenylhydroxylamine in the Rat[1]

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SUMMARY

The *O*-glucuronide of *N*-hydroxy-2-acetylaminofluorene was assayed for carcinogenicity by s.c. injection in female Holtzman rats. No tumors were produced in animals in which tricaprylin was used as a vehicle for administration of *N*-hydroxy-2-acetylaminofluorene-*O*-glucuronide as a suspension. However, when injected s.c. as a solution in 0.9% NaCl, *N*-hydroxy-2-acetylaminofluorene-*O*-glucuronide induced a total of nine tumors in 16 rats: one sarcoma at the site of injection, four mammary carcinomas, three ear duct carcinomas, and one liver carcinoma. The influence of solvent for assay of carcinogenicity was demonstrated also in experiments with tricaprylin and 0.9% NaCl as vehicles for administration of the aglycone, *N*-hydroxy-2-acetylaminofluorene. When *N*-hydroxy-2-acetylaminofluorene was given as a suspension in tricaprylin, 35 tumors were produced in 16 rats, whereas, when it was administered as a suspension in 0.9% NaCl, 20 tumors were found in 16 rats. Only two tumors, a sarcoma at the site of injection and a mammary carcinoma (both in the same rat), were found in 16 rats that received s.c. injections of the *N*-2-fluorenylhydroxylamine-*O*-glucuronide.

INTRODUCTION

The glucuronide of N-HO-AAF[2] is a major metabolite of AAF and N-HO-AAF in species which are susceptible to these carcinogens (4, 15). N-GlO-AAF reacts *in vitro* with methionine, tryptophan, and guanosine and with proteins and nucleic acids containing these nucleophiles, although the rate of reaction is much lower than that observed for N-acetoxy-AAF and the *O*-sulfonate of N-HO-AAF (4, 8, 9, 13, 15). Preliminary testing of N-GlO-AAF for carcinogenicity by s.c. injection in female rats revealed that the compound was only weakly carcinogenic (Ref. 13; see also Ref. 10, Footnote 3, for additional data). N-GlO-AAF is a highly polar conjugate, negatively charged at physiological pH, and is extremely water soluble. Since the preliminary carcinogenicity assays were carried out with tricaprylin as a solvent, we wanted to repeat these tests, using an aqueous vehicle for the injections. Solvent effects may be pronounced and very often do have a major influence on carcinogenesis (1, 17).

The synthesis and characterization of N-GlO-AF, a potential metabolite of N-HO-AAF, was described recently (5). N-GlO-AF is quite unstable in aqueous systems and reacts with guanine residues of RNA and DNA *in vitro* at a rate much faster than does N-GlO-AAF. Furthermore, the mutagenic activity of N-GlO-AF to *Bacillus subtilis*-transforming DNA equals or surpasses the mutagenicity of N-acetoxy-AAF and the *O*-sulfonate of N-HO-AAF (12), whereas N-GlO-AAF was not mutagenic in this system (11) or in the T4 bacteriophage system (2). Consequently, it seemed desirable to test N-GlO-AF for carcinogenicity by s.c. injection in female rats.

MATERIALS AND METHODS

N-HO-AAF and N-HO-AF were synthesized by published methods (16). We prepared N-GlO-AAF biosynthetically by feeding N-HO-AAF to rabbits and isolating the glucuronide from the urine (3). The glucuronide was obtained as the crystalline sodium salt. The sodium salt of N-GlO-AF was synthesized from either N-GlO-AAF or the triacetyl methyl ester derivative of N-GlO-AAF (5). Tricaprylin (trioctanoin) was purchased in a single lot from Distillation Products Industries, Rochester, N. Y.

Young female rats with initial weights of 110 to 130 g were obtained from the Charles River Breeding Laboratories, North Wilmington, Mass. The animals were housed in individual cages and were fed Purina laboratory chow and water *ad libitum*. Three times weekly (on Monday, Wednesday, and Friday), each rat received a 0.2-ml s.c. injection, of either: (*a*) 0.9% NaCl solution; (*b*) tricaprylin; (*c*) 5.7 mg (12.5 μmoles) of N-GlO-AAF in 0.9% NaCl; (*d*) 5.7 mg of N-GlO-AAF in tricaprylin; (*e*) 5.0 mg (12.6 μmoles) of N-GlO-AF in tricaprylin; (*f*) 3.0 mg (12.5 μmoles) of N-HO-AAF in 0.9% NaCl; (*g*) 3.0 mg of N-HO-AAF in tricaprylin; or (*h*) 2.5 mg (12.7 μmoles) of N-HO-AF in tricaprylin. With the exception of N-GlO-AAF, all compounds were injected as suspensions. N-GlO-AAF dissolved readily in 0.9% NaCl, but not in tricaprylin, and was injected as a suspension in this vehicle. We prepared suspensions of the compounds immediately prior to

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[2] The abbreviations used are: N-HO-AAF, *N*-hydroxy-2-acetylaminofluorene; AAF, 2-acetylaminofluorene; N-GlO-AAF, *O*-glucuronide of N-HO-AAF; N-GlO-AF, *O*-glucuronide of *N*-2-fluorenylhydroxylamine.

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Table 1

Assay of carcinogenicity of N-GlO-AAF, N-GlO-AF, and their aglycones by repeated s.c. injections in female rats

The animals received injections s.c. 3 times weekly of vehicle alone or vehicle containing 12.5 to 12.7 μmoles of compound. Each rat received 28 injections.

Compound injected	Vehicle	Mean initial body weight (g)	Mean weight gain at 2 mo. (g)	Survival[a] at 2 mo.	Survival[a] at 13 mo.	Incidence of tumors at 13 mo.[b]	Distribution of tumors: Sarcomas at injection site	Distribution of tumors: Mammary carcinoma	Distribution of tumors: Ear duct gland carcinoma
None	0.9% NaCl solution	121	141	16/16	14/16	0/16	0	0	0
None	Tricaprylin	119	149	16/16	16/16	0/16	0	0	0
N-GlO-AAF	0.9% NaCl solution	116	122	16/16	11/16	8/16[c]	1	4	3
N-GlO-AAF	Tricaprylin	118	132	16/16	16/16	0/16	0	0	0
N-GlO-AF	Tricaprylin	123	117	16/16	15/16	1/16	1	1	0
N-HO-AAF	0.9% NaCl	121	105	16/16	1/16	15/16	8	9	3
N-HO-AAF	Tricaprylin	117	106	16/16	0/16	16/16	10	12	13
N-HO-AF	Tricaprylin	121	86	9/16	1/16	5/9	5	0	1

[a] Survival is expressed as no. of rats alive/no. of rats at start of experiment.
[b] Accumulated incidence of tumors at 13 months expressed as no. of rats with tumors/no. of rats surviving at 2 months.
[c] One poorly differentiated carcinoma of the liver, in addition to tumors tabulated.

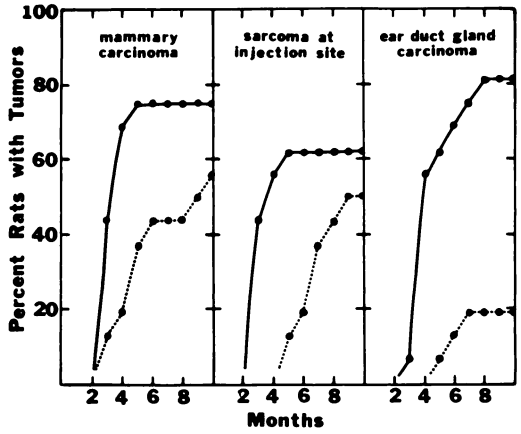

Chart 1. Influence of solvent on the carcinogenicity of N-HO-AAF. Female rats were given s.c. injections of N-HO-AAF suspended in tricaprylin (•- - -•) or in 0.9% NaCl solution (•- - -•) for 9 weeks, as described in "Materials and Methods."

injection by grinding the compounds in the appropriate vehicle in a glass homogenizer, using a Teflon pestle. The rats received 28 injections over a period of 9 weeks; the total dose of each compound injected was 0.35 mmole. All animals were treated periodically with Terramycin (Pfizer Laboratories, New York, N. Y.) in the drinking water (75 mg/liter). At intervals of 5 weeks, water was withheld from the rats for 1 day, followed by 2 days on Terramycin, 1 day without water and, finally, 2 days with Terramycin again. The rats were weighed each week, at which time they were palpated for tumors. At death, or termination of the experiment, a necropsy was performed on each animal. All gross tumors or other abnormal tissues were fixed in neutral 10% formalin, sectioned at 6 to 8 μ, and stained with hematoxylin and eosin. We are grateful to Dr. Joseph Young, Laboratory Service, Veterans Administration Hospital, for the histological diagnoses.

RESULTS

The experiment was terminated 20 months after the injections were started. However, tumor incidence was tabulated at 13 months, since no carcinomas or sarcomas were found in any of the rats after 13 months. By 20 months, a few of the animals had benign mammary tumors, but these occurred with equal frequency in the control rats and in the surviving rats in the other groups. At 13 months, no tumors were found in any of the control animals given either 0.9% NaCl solution or tricaprylin (Table 1).

There were no tumors by 13 months in any of the rats given N-GlO-AAF in tricaprylin. These data are in agreement with those from the previous preliminary testing of this compound in tricaprylin, in which no tumors were produced at 12 months by s.c injection of slightly lower doses of N-GlO-AAF (13). The data differ, however, in that, in the earlier report, 3 of 16 rats later developed sarcomas at the injection site (see Ref. 10, Footnote 3). On the other hand, when 0.9% NaCl solution was used as the vehicle for administration of the N-GlO-AAF, 50% of the animals developed tumors: 1 with a sarcoma at the site of injection, 4 with mammary carcinomas, and 3 with ear duct carcinomas. In addition, 1 of these rats had a poorly differentiated carcinoma of the liver.

Because of the instability of N-GlO-AF in aqueous systems (5), this compound could not be tested with 0.9% NaCl solution as a vehicle. When the N-GlO-AF was given in tricaprylin, only 1 of 16 rats developed a sarcoma at the injection site (Table 1). The same rat also had a mammary carcinoma. There were no other tumors in this group of animals.

As controls, we also tested the aglycones of these 2 glucuronides, *i.e.*, N-HO-AAF and N-HO-AF, by s. c. injection. As reported previously (13), N-HO-AAF was much more carcinogenic than its glucuronide metabolite (Table 1). However, when given in 0.9% NaCl solution, N-HO-AAF was

less carcinogenic than when given in tricaprylin. Although there was no difference in the number of rats with tumors, there was a total of only 20 tumors in the group in which 0.9% NaCl solution was used as the vehicle, compared with 35 tumors in the group in which tricaprylin was used (Table 1). Furthermore, as illustrated in Chart 1, the mean latent period of appearance of all tumors was greater in the rats given N-HO-AAF as a suspension in 0.9% NaCl: mammary gland, 5.9 months for 0.9% NaCl solution, 3.5 months for tricaprylin; sarcoma at the injection site, 7.1 months *versus* 3.5 months; and for ear duct gland carcinoma, 6.0 months *versus* 4.7 months.

N-HO-AF proved to be the most toxic of the compounds injected, with only 56% of the animals surviving the 2-month period of injections (Table 1). In the rats that did survive the 2 months, the incidence of sarcomas at the site of injection was about the same as that obtained after s.c. administration of N-HO-AAF. However, in contrast to results obtained with N-HO-AAF, only 1 tumor was obtained at a remote site (the ear duct) following the s.c. injection of N-HO-AF.

DISCUSSION

As pointed out before, the most readily interpretable data on the carcinogenicity of N-GlO-AAF would be obtained by the induction of sarcomas at the site of s.c. injection (10). The results of the present studies, taken together with data previously reported in collaboration with E. C. Miller and J. A. Miller (10, 13), indicate that, when tested by s.c. injection in the doses used, N-GlO-AAF is carcinogenic (total of 4 sarcomas in 32 rats), albeit weak under these conditions. However, in view of the rapid absorption and excretion of N-GlO-AAF following s.c. injection (10) and the anionic character of the glucuronide, which may hinder penetration into cells (15), we feel that even a low incidence of tumors at the injection site is significant. On the other hand, the possibility that the observed low tumor incidence could be due to enzymatic hydrolysis of N-GlO-AAF to N-HO-AAF at the injection site cannot be excluded. Upon s.c. injection in 0.9% NaCl solution, but not in tricaprylin, N-GlO-AAF induced tumors in 50% of the rats tested. Most of these tumors were in tissues distant from the injection site—the mammary gland and the ear duct—known to be susceptible to AAF and N-HO-AAF, as well as to a number of other carcinogens. Interpretation of data on the induction of tumors in remote tissues may be complicated by the fact that N-GlO-AAF is rapidly excreted in the bile and is metabolized in the intestinal tract by the bacterial flora to N-HO-AAF and AAF (18). Enterohepatic circulation of these metabolites ensues.

Results obtained by testing N-GlO-AAF and N-HO-AAF in both tricaprylin and 0.9% NaCl offer further documentation that the choice of solvent may have great influence on the outcome of an assay of a compound for carcinogenic activity, particularly when tested by s.c. injection.

N-GlO-AF, an extremely reactive and unstable compound, proved to be marginally carcinogenic by s.c. injection in tricaprylin. Problems inherent in the testing of such unstable, water-soluble, anionic derivatives of carcinogens have been mentioned by others (15). The high reactivity of these derivatives may cause much of the applied dose to be expended on extracellular or noncritical cellular nucleophiles (15).

Attention has already been drawn to the fact that N-GlO-AAF is a major metabolite of AAF and N-HO-AAF in animals in which these compounds induce cancer. The reactivity of this glucuronide with tissue nucleophiles has been reviewed (4), and on the basis of our studies we have proposed that N-GlO-AAF plays an essential role in the binding of AAF and N-HO-AAF to rat liver DNA *in vivo* (4, 6, 7, 9). Since no relationship has been established between the binding of carcinogens to liver DNA and hepatocarcinogenesis, we cannot conclude that N-GlO-AAF is involved in the induction of liver cancer by AAF or N-HO-AAF. To the contrary, Miller *et al.* (14) stated that the high levels of N-GlO-AAF formed by female rats, by thyroidectomized male rats, and by mice, hamsters, and rabbits, all of which are relatively resistant to hepatocarcinogenesis by AAF, do not support the concept that this glucuronide has a major role in hepatocarcinogenesis. However, at the same time, we would point out that such data alone do not provide any evidence that N-GlO-AAF is not implicated in the mechanism of hepatocarcinogenesis by AAF. Just how the binding of 2-aminofluorene and AAF residues to liver DNA is involved in hepatocarcinogenesis and how other factors—such as the proliferation which is induced by the hepatotoxic action of the carcinogen—might influence the consequence of binding of 2-aminofluorene and AAF residues to liver DNA, remain speculative at this point. In the meantime, as reported herein, further efforts to demonstrate the carcinogenicity of N-GlO-AAF have been made. We feel that there are now sufficient data at hand to indicate that this glucuronide is carcinogenic in the rat.

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